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Award Number: W81XWH-05-1-0054

TITLE: Mitochondrial DNA Mutations in Epithelial Ovarian Tumor Progression

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REPORT DATE: December 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                         Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                            Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
01-12-2007	Annual	1 DEC 2006 - 30 NOV 2007

4. TITLE AND SUBTITLE

Mitochondrial DNA Mutations in Epithelial Ovarian Tumor Progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0054

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Morehouse School of Medicine
Atlanta, GA 30310-1495

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Early diagnosis is one of the greatest challenges facing clinicians in the treatment of epithelial ovarian cancer. As a result of mitochondria role in apoptosis and ROS, it have been implicated in carcinogenesis. In this study we detected sequence variants in two fragments of mitochondrial DNA obtained from 68 epithelia ovarian cancer tissues spanning 5317 to 7608 and 8282 to 10110 base pair, including NADH subunits 2, 3, CO I,II,III, part of ATPase 6 and several tRNA genes. MtDNA variants were obtained by using methods of RFLP and PCR-based sequence and analyzed in relationship to ovarian tumor subtypes/stages, ages and races of the patients. Thirty-nine polymorphisms/mutations were detected of which 28 were unreported. Two variant C7256T and G7520A showed a frequency of 45% (5/11) in endometriod stage III while has no incidence in serous or mucinous subtype stage III was observed. An unreported polymorphism at T8548G in ATP6 gene was detected at a high frequency of 92% in ovarian serous subtype tissues in stages II-IV. In addition, an unreported mutation at np C7520T in tRNA gene occurred in 73% African American and 27% white samples. Interestingly, variants C7020T (56%) and at np A8860G (92%) were evenly distributed in all three studied ovarian tumor subtype and stages. Perhaps, it is tempting to speculate that variant A8860G that we observed in our study may play a potential role in the onset and progression of epithelial ovarian tumor subtypes and stages.

15. SUBJECT TERMS
Epithelial Ovarian Cancer, Mitochondrial DNA

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
U	U	U	UU	19	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

Ovarian cancer is characterized by few early symptoms and diagnosed at an advanced stage leading to poor survival [1]. Most ovarian cancers occur after menopause when the ovaries have no physiological role and combined with anatomical location of the ovaries deep in the pelvis, ovarian cancers typically cause few symptoms until they reach a large size or have disseminated. Despite advances in surgical and chemotherapeutic management during the last decade, the survival rates are poor. Almost 80-90% of the patients that are diagnosed with metastatic disease in the pelvis or abdomens have five-year survival rates [2]. In contrast, the small proportion of patients diagnosed with stage I ovarian cancer (confined to the ovaries) have a five-year survival rate in excess of 90% [1]. Ovarian cancer pre-malignant precursor lesion has not been fully identified and, this limits the focus of screening techniques development to detection of asymptomatic, early stage disease [1,3]. The histologic classification of ovarian carcinomas is based on morphological criteria and corresponds to the different types of epithelial in the female reproductive system, including serous, mucinous, endometrioid, clear cell and Brenner. Each has been further sub-classified into benign, malignant, and borderline to reflect their histopathology. Serous ovarian carcinoma represents the major histological subtype of ovarian cancer and is the most lethal gynecologic malignancy. The relationship between stage at presentation and survival in serous ovarian cancer has long provided a rationale for efforts to improve outcomes by development of the screening techniques for detection of early stage disease and to determine the changes that occur during serous ovarian tumorigenesis. During the last decade, research efforts have been directed toward improving outcomes for ovarian cancer by screening for pre-clinical, early stage disease using both imaging techniques and serum markers. Numerous biomarkers have shown potential for screening samples from clinically diagnosed ovarian cancer patients [4], but few have been able to thoroughly assess preclinical disease. The CA 125 and the transvaginal ultrasound are the most thoroughly investigated biomarker and screening techniques for ovarian cancer [4,5]. However, while an elevated CA 125 level indicates a probability of epithelial ovarian cancer in postmenopausal women, a negative CA 125 level cannot be used to exclude the presence of residual disease. Moreover, most pre-menopausal women without epithelial ovarian cancer usually exhibit high CA 125 levels and other malignancies can also elevate CA 125 levels [6]. Therefore, the best evaluation of epithelial ovarian cancer progression, such as the early serous adenocarcinoma, continues to confound clinicians. **Thus, the development of novel molecular methodologies are critically needed to facilitate early detection of epithelial ovarian cancer**.

Human mitochondrial gene mutations have increasingly been associated with various cancers with unclear pathophysiological significance [3,5-,9]. Most mtDNA mutations occur in coding sequences because mtDNA lacks introns. The mitochondrial genome is more vulnerable to oxidative damage and undergo a higher rate of mutation than does the nuclear genome [10,11]. Some of the mitochondrial genome aberrations found in cancer tissues are mutations in mtDNA-encoded Complexes I, III-V as well as mutations in the hypervariable regions [9]. MtDNA mutations in tumor cells are consistent with previous reports that tumor cells are subject to constitutive oxidative stress [9,11,12]. Although most of the reported mtDNA mutations in cancer are homoplasmic polymorphisms which are considered too subtle to cause any effect on oxidative phosphorylation, (OXPHOS) long-term accumulation of subtle difference in OXPHOS activity may eventually result in oxidative stress [12,13]. Consequently, variations in the mtDNA may potentially play a role as a modifying risk factor in the development of age related diseases such as ovarian cancer. Mitochondrial aberrations have been identified in tumors of colorectal, breast, head and neck, kidney, lung, stomach and in the hematologic malignancies, leukemia and lymphoma [7,13-20]. Hence, we sought to determine the impact of mtDNA sequence variants in the pathogenicity of epithelial ovarian tumor subtypes.

Here we report the identification of mtDNA sequence variants in multiple specimens of ovarian carcinoma using a high-resolution restriction endonucleases and PCR-based sequencing analyses. We have found several highly frequent mtDNA sequence variants that could differentiate the three major histopathological subtypes and stages of serous, endometrioid and mucinous carcinomas of the ovary.

BODY
APPROVED STATEMENT OF WORK

Task 1. To identify mtDNA mutations/polymorphisms among and within stages of epithelial ovarian cancer, focusing on serous, mucinous and endometrioid subtypes (1-18 Months).

a. Collections and selections of paraffin embedded/frozen tissues of appropriate ovarian tumor subtypes/stages for the first phase of the study from Grady Hospital Pathology and UAB-Tissue procurement divisions (1-3 months).
b. Sectioning of the paraffin embedded tissues and extraction of genomic DNA samples from both paraffin and frozen tissues. PCR amplification, and attempt to optimize the utility of WAVE^{TM} Technology for rapid screening of the extracted DNA. (3-7 Months).

c. PCR product purifications, restriction analysis and restriction site variant scoring (7-12 Months).
d. Collections and selections of more tissues, sectioning, extractions, amplifications, purifications,   restriction analyses will be ongoing for months (1-30 months).
e. Sequence analyses and identifications of specific polymorphisms and mutations (12-18 months).

Task 2. To evaluate the effect of stage specific mtDNA mutations/polymorphisms in epithelial ovarian tumor progression and determine the strengths and limitations in detection, and screening for ovarian cancer. Also, determine if there is an overall significant difference in the frequency of one or more variants in ovarian cancer samples vs. non-cancerous tissues (18-36 Months).

a. Confirm that fragments of the sequence polymorphisms /Mutations are subtypes and stages specific of ovarian cancer (18-24 Months).
b. Statistical analyses of sequences among subtypes/stages variations with association testing (24-28 Months).
c. Test the effect of sequence variants by performing feasibility experiments to assess fresh ovarian tumors (30-36 Months).

Please note that we have summarized an anticipated timeline but the work-load will be modified so as to maximize the efficiency of achieving the tasks.

DATA PRESENTATION AND INTERPRETATION OF CURRENT RESULTS
 A significant portion of our work over the past 12 months has been directed at completing Task 2. **To evaluate the effect of stage specific mtDNA mutations/polymorphisms in epithelial ovarian tumor progression and determine the strengths and limitations in detection, and screening for ovarian cancer. Also, determine if there is an overall significant difference in the frequency of one or more variants in ovarian cancer samples vs. non-cancerous tissues (18-36 Months).**
a. Up-to-date, we have collected a total of 255 frozen/paraffin of ovarian tumor tissue samples.
b. We have done analyses on 185 out of 255 of the collected ovarian tumor tissue samples have been PCR-amplified using the entire mitochondrial genome 9 overlapping primer sets, purified and analyzed by high-resolution restriction analyses and mtDNA sequencing.
c. We have completed the sequencing of mtDNA that spanned of 9.3 kb fragment, including D-Loop, 12S rRNA-tRNA^{phe}, tRNA^{val,} *COX I*, tRNA^{ser} , tRNA^{asp}, *COX II*, tRNA^{lys}, *ATPase 6*, *ATPase 8, COX III, ND2, and ND3 genes*
d. We have identified certain mtDNA sequence variants associated with ovarian tumor subtypes and stages.
e. We identify high frequencies of mtDNA mutations G7520A (67%) and T9540C (88%) among African American ovarian cancer tissues compare to 7% and 10% among White ovarian cancer tissues respectively.
f. We have observed mtDNA mutation A8860G (92%) that was evenly distributed in all three studied ovarian tumor subtype and stages.
The above tasks have been accomplished as proposed.

Results and Discussion

Sequence analysis of the two fragments of mitochondrial DNA spanning from 5317 to7608 and 8282 to 9031 base pairs that were obtained from 68 epithelia ovarian cancer tissues revealed the presence thirty-nine polymorphisms of which 28 unreported (Table1). The observed mutations with notably frequencies (41-93%) among these samples were at np C7028T,   C7256T, G7520A, T8548G, T8588C, A8860G, C9488G, C9500T, T9540C, C9857T, and T9951C. Furthermore, six unreported point mutations with frequencies of 14-41% were observed at np G7520A, T8548G, C9488G, C9500T, C9857 and T9951C. A combined mutation of G7520A and C7256T was frequent at 45% (5/11) in endometriod stage III only. Interestingly, variants C7020T (56%) and at np A8860G (92%) were evenly distributed in all three studied ovarian tumor subtype and stages. However, a variant G –to-A at np 8860 has been previously reported in a general population that was considered as a "control group" [21]. Perhaps, it is tempting to speculate that variant A8860G that we observed in our study may play a potential role in the onset and progression of epithelial ovarian tumor subtypes and stages.

The most striking is the age and race related distribution of C7028T mutation that was observed in 55% of studied samples that was evenly distributed in all age groups (75%, 33%, 78%, 50%, 73%, in age groups of 31-40, 41-50, 51-60, 61-70 and over 71 respectively). The frequency of this variant C7028T was higher in African American patients (83%) compare to white patients (49%) in all age groups (Table 2). The frequency of this mutation at the age under 40 years was almost twice in African American patients (27%) compare to white patients (15%). In addition, the unreported mutation at np C7520T, at tRNA gene occurred in 73% African American and 27% white samples. A mutation of A8860G variant was detected in 92% of all tested samples of which 94% in white and 100% in African-American samples. Also, this mutation was observed in 68% of African American patients under age 40 and only 9% of white under age 40. However, at age over 60, this mutation was detected in 53% white compare to 33% of African American samples. Variants T9540C and C7520T showed significant higher frequency in African American samples compare to white samples and these may be used for further investigation for cancer disparity study (Table 2).

The high frequency of mutations in human mitochondrial DNA (mtDNA) in older people have led to speculation that mtDNA mutations might contribute to aging or accumulate in postmitotic tissues with age [22] and Mitochondria DNA mutations in thyroid cancer with respect to age factors has been reported. [23, 24]. Ovarian cancer is relatively age related disease. Most ovarian cancers occur after menopause when the ovaries have no physiological role and combined with anatomical location of the ovaries deep in the pelvis, ovarian cancers typically cause few symptoms until they reach a large size or have disseminated. Correlation of these mtDNA variants in ovarian tumor subtypes and stages in this study merit further investigation in a larger population, which may be needed to elucidate fully the significance of these mtDNA variants role in ovarian tumorigenesis.

Table 1. **Mutations frequencies in three epithelial ovarian subtypes**

Mt region/genes subtype	Nucleotide position np	Mutation frequency	% frequency in		
			Serous	Endometriod	Mucinous
MT-CO1 Reported	C7028T	55	54	56	54
MT-CO1 Reported	C7256T	13	0	22	23
MT-TD New	G7520A	16	7	30	15
MT-ATP New	T8548G	35	34	25	50
MT-ATP6 New	T8588C	17	16	13	33
MT-ATP6 Reported	A8860G	92	95	88	100
Mt-CO3 New	G9488G	31	36	25	33
MT-CO3 New	C9500T	40	47	25	16
MT-CO3 Reported	T9540C	21	24	25	0
MT-CO3 New	C9457T	25	18	38	50
MT-TG	A de(10045-10046)	29	34	12	16

Table 2. Mutations race related distribution

Region	Total frequency	Frequency in black	Frequency in white	
C7028T	55	83	49	
C7256T	13	67	33	
G7520A	16	67	7	
T8548G new	35	37	31	
A8860G	92	100	92	
C9488G new	31	25	24	
C9500T	40	50	32	
T9540C reported.	21	88	10	
C9857T New	25	25	20	
T de 10045-10046 new	29	38	30	
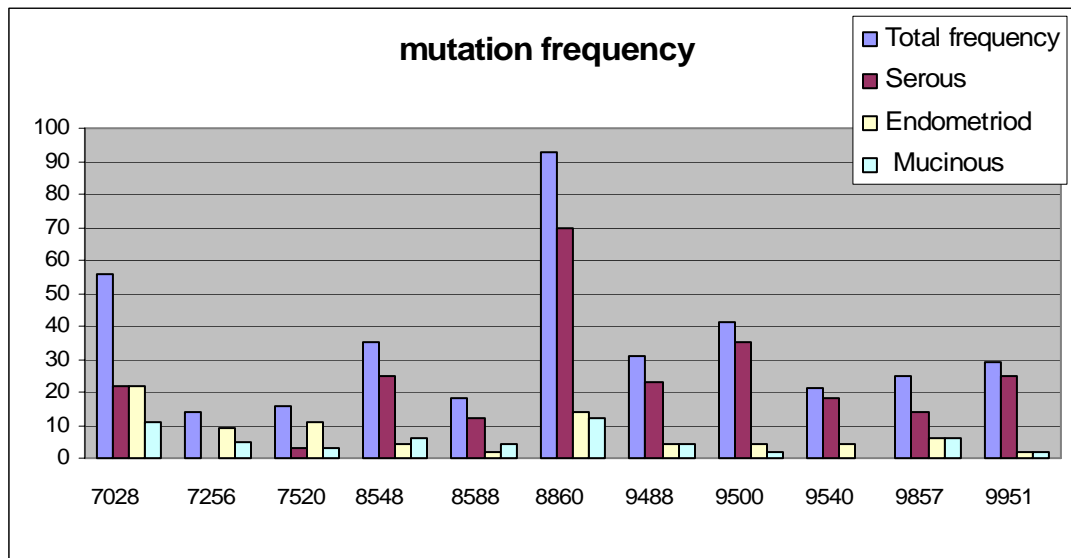
Fig.1. Distributions of the most frequent mtDNA mutations in the three epithelial ovarian tumor subtypes and stages. Mutation A8860G appears to be most frequent in the ovarian tumors.

PROPRIETARY & UNPUBLISHED DATA

KEY RESEARCH ACCOMPLISHMENTS

* We have define most of mitochondrial genome of each sample by PCR amplification using 9 overlapping primer sets and sequenced a span of 9.3 kb fragment, including D-Loop, 12S rRNA- tRNA^{phe}, tRNA^{val,} COX I, II, III, tRNA^{ser} , tRNA^{asp}, COX II, tRNA^{lys}, *ATPase 6*, *ATPase 8 and ND genes*

* We identify high frequencies of mtDNA mutations G7520A (67%) and T9540C (88%) among African American ovarian cancer tissues compare to 7% and 10% among White ovarian cancer tissues respectively.

*We have observed mtDNA mutation A8860G (92%) that was intriguingly even distributed in all three studied ovarian tumor subtype and stages.

REPORTABLE OUTCOMES

One abstract/ poster presentation at **98th American Association for Cancer Research at Los Angeles, CA.** Proc Amer Assoc Res 2007 ;48 : 3532 (see appendix)
-Title of the presentation: MtDNA D310insTC variant in tumorigenesis of ovarian serous carcinoma.
One published manuscript: Aikhionbare, FO, Mehrabi, S, Kumaresn, K, Zavareh, M, Olatinwo M, Odunsi, K, Partridge E. Mitochondrial DNA sequence variants in epithelial ovarian tumor subtypes and stages. J. of Carcinogenesis. 6:1 doi;10.1186/1477-3163-6-1 2007. (see appendix).

CONCLUSIONS

Our study demonstrates that mitochondrial DNA sequence variants, such as A8860G (92%) that were evenly distributed in all three studied ovarian tumor subtype and stages, which occurred in *ATPase 6 gene* region that potentially influence the ROS productions, may play a potential role in the onset and progression of epithelial ovarian tumor subtypes and stages. Although our study lacks functional data and clinical outcome of the mitochondrial genome alterations in relationship to ovarian neoplasms, it suggests that mtDNA sequence variants may play a role in etiological differences that may exist between the pathogenicity of subtypes and stages of benign and invasive epithelial ovarian tumors. Large population-based studies are required to precisely quantify the functional role of mtDNA sequence variants in histological subtypes and stages of ovarian cancer. Given that we sequenced 9.3 kb of 16.5 kb fragments of mitochondrial genome, the level of mtDNA sequence variants that could be correlated with ovarian tumor subtypes and stages may far exceed the number we have observed in this current results. **Future plan**; We will be sequencing the remaining regions of the 7.2 kb of the mtDNA among the collected samples during the coming months, and to submit a manuscript to Cancer Research Journal. To write a grant proposal to be submitted to NIH or DOD to continue the functional aspect of the project.

PROPRIETARY & UNPUBLISHED DATA

Reference

1. Jemal A, Murray, T, Ward E, at al. Cancer Statistics, 2005 CA Cancer J Clin 2005; 55:10-30.
2. American Cancer Society.: Cancer Facts and Figures 2004. Atlanta, GA:American Cancer Society, 2004
3. Menon, U., Jacob I J. The current Status of Screening for Ovarian Cancer. In Ovarian Cancer (Jacob, IJ Shepherd, JH, Oram, DH Blackett, AD, Luesley, DM., Berchuck A, and Hudson CN, eds) pp. 171-178, Oxford University Press, London
4. Zhang, Z. et al. Three Biomarkers identified from Serum Proteomic Analysis fro the Detection of Early Stages\Ovarian Cancer Cancer Research 2004; 64 1-7.
5. Bell R, Petticrew M, Luengo S, Sheldon TA. Screening for ovarian cancer: a systemic review Health Technol Assessment 1998; 2(2) 1-4
6. Obata, K., S.J. Morland, R.H.Watson, A. Hitchcock, G. Chenevix-Trench, E.J.Thomas and I.G. Campbell. Frequent PTEN/MMAC mutations in endometrioid but not serous or mucinous epithelial ovarian tumors. *Cancer Res* 58: 2095-2097, 1998.
7. Aikhionbare FO et al:.: **Is cumulative frequency of mitochondrial DNA variants a biomarker for colorectal tumor progression?** Molecular Cancer. 2004; DOI:10.1186/1476-4598-3-30.
    Penta JS et al.:**Mitochondrial DNA in human malignancy**. Mutat Res . 2001; 488: 119-133.
9. Yakes FM, Van Houten B.: **Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress**. Proc Natl Acad Sci USA. 1997; 94:514-9.
    Marcelino LA, Thilly WG.:**Mitochondrial mutagenesis in human cells and tissues.** Mutat Res.1999; 434:177-203.
11. Toyokuni S, Okamoto K, Yodoi J, Hiai H.: **Persistent oxidative stress in cancer.** FEBS lett. 1995; 358:1-3.
12. Polyak K,et al.: **Somatics mutations of the mitochondrial genome in human colorectal tumours**. Nat. Genet. 1998; 20:291-293.
13. Richard SM,et al.: **Nuclear and mitochondrial genome instability in human breast cancer**. Cancer Res. 2000; 60: 4231-7.
14. Tamura G,et al.: **Mutations in mitochondrial control region DNA in gastric tumuors of Japanese patients.** Eur J cancer . 1999; 35:316-319.
16 Ha PK,et al.: **Mitochondrial C-tract alternation in premalignant lesions of the head and neck. a marker for progression and clonal proliferation.** Clin Cancer Res. 2002; 8 : 2260-2265.
15. Nagy A, Wilhelm M, and Kovacs G.: **Mutations of mtDNA in renal cell tumours arising in end-stage renal disease.** J Pathol.2003; 199: 237-242.
18 Matsuyama W,et al.: **Mitochondrial DNA mutation correlates with stage progression and prognosis in non-small cell lung cancer.** Hum Mutat. 2003; 21:441-443
19 Vega A et al.: **mtDNA mutations in tumors of the central nervous system reflect the neutral evolution of mtDNA in populations.** Oncogene. 2004; 23:1314-1320.2
20 Fliss MS et al.: **Facile detection of mitochondrial DNA mutations in tumors and bodily fluids.** Science.2000; 287: 2017-2019
21. Polyak, Kornelia ; Vogelstein, Bert , 1999. MITOMAP mtDNA Sequence Data, /cgi-bin/tbl15gen.pl#19990316019
22. Raule N, Sevini F, Santoro A, Altilia S, Franceschi C. Association studies on human mitochondrial DNA: methodological aspects and results in the most common age-related diseases. Mitochondrion 2007 (1-2)7:29-83
23. Modica-Napolitano JS, KulawiecM, Singh KK. Mitochondria and Human cancer. Curr Mol Med. 2007 7(1):121-31
    24. Baysal BE . Role of mitochondrial mutations in cancer.Endocr Pathol. 2006 17(3):203-12

2007 AACR Annual Meeting
April 14-18, 2007
Los Angeles, CA

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Abstract Number:	3532
Presentation Title:	**MtDNA D310insTC variant in tumorigenesis of ovarian serous carcinoma**
Presentation Start/End Time:	Tuesday, Apr 17, 2007, 8:00 AM -12:00 PM
Location:	Exhibit Hall, Los Angeles Convention Center
Poster Section:	7
Poster Board Number:	23

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Approximately 80-90% of ovarian tumors arise from the surface epithelium of the ovary and the serous tumors are by far the most common subtype of the epithelial ovarian tumors. Although old age and a positive family history are associated risk factors, the molecular events that underlie the development and progression of the various subtypes and stages of epithelial ovarian cancer is not completely understood. The goal of this study was to assess the significance of the mitochondrial DNA sequence variants associated with the different histologic subtypes and stages of epithelial ovarian cancer.

A total of 102 frozen epithelial ovarian tumor tissue samples of serous, endometrioid and mucinous subtypes and stages were selected based on the criteria outlined by the International Federation of Gynecology and Obstetrics (FIGO). High-resolution restriction endonucleases and PCR-based sequencing analysis were used to assess the mtDNA sequence variants spanning 3.3 kb fragment that comprised the D-Loop, 12S rRNA- tRNA[phe], tRNA[val,], tRNA[ser] , tRNA[asp], tRNA[lys], *ATPase 6 ATPase 8, cytochrome oxidase I and II* genes.

Notably, the incidence of mtDNA instability at np 303-315 were found in 97% of our study samples. Interestingly, a high frequency, 81% (13/16) of TC insertion at np 310 was found only in cystadenomas, 3/3; borderline, 4/4; stage IA, 2/2; stage IB, 0/3 and matched normal tissues, 4/4 of the ovarian serous subtype. Our findings suggest that mitochondrial DNA sequence variant, TC insertion at np 310, in combination with the mtDNA instability at np 303-315, may play a potential role in the tumorigensis of ovarian serous carcinoma.

2007 AACR Annual Meeting
April 14-18, 2007
Los Angeles, CA

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Journal of Carcinogenesis

Research

Mitochondrial DNA sequence variants in epithelial ovarian tumor subtypes and stages

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Abstract

Background: A majority of primary ovarian neoplasms arise from cell surface epithelium of the ovaries. Although old age and a positive family history are associated risk factors, the etiology of the epithelial ovarian tumors is not completely understood. Additionally, knowledge of factors involved in the histogenesis of the various subtypes of this tumor as well as those factors that promote progression to advanced stages of ovarian malignancy are largely unknown. Current evidence suggests that mitochondrial alterations involved in cellular signaling pathways may be associated with tumorigenesis.

Methods: In this study, we determined the presence of polymorphisms and other sequence variants of mitochondrial DNA (mtDNA) in 102 epithelial ovarian tumors including 10 matched normal tissues that paired with some of the tumors. High-resolution restriction endonucleases and PCR-based sequencing were used to assess the mtDNA variants spanning 3.3 kb fragment that comprised the D-Loop and 12S rRNA-tRNA^{phe}, tRNA^{val}, tRNA^{ser}, tRNA^{asp}, tRNA^{lys}, *ATPase 6, ATPase 8, cytochrome oxidase I and II* genes.

Results: Three hundred and fifty-two (352) mtDNA sequence variants were identified, of which 238 of 352 (68%) have not been previously reported. There were relatively high frequencies of three mutations in the 12S rRNA gene at np 772, 773, and 780 in stage IIIC endometrioid tumors, two of which are novel (773delT and 780delC), and occurred with a frequency of 100% (7/7). Furthermore, two mutations were observed in serous tumors only at np 1657 in stage IV (10/10), and at np 8221delA in benign cystadenomas (3/3) and borderline tumors (4/4). A high frequency, 81% (13/16) of TC insertion at np 310 was found only in early stages of serous subtype (benign cystadenomas, 3/3; borderline tumors, 4/4; stage I tumors, 2/5 and matched normal tissues 4/4).

Conclusion: Our findings indicate that certain mtDNA mutations can reliably distinguish the different histologic subtypes of epithelial ovarian tumors. In addition, these data raise the possibility that certain mtDNA mutations may be useful biomarkers for predicting tumor aggressiveness and may play a potential role in tumorigenesis.

Background

Epithelial ovarian cancer is the fifth leading cause of cancer mortality among women in the United States [1]. The majority (80–90%) of benign and malignant ovarian tumors originate from the surface epithelium, even though all cell types of the human ovary may undergo neoplastic transformation [2,3]. Most of the histopathological differences that are reflected as serous, endometrioid, mucinous, clear cell, and transitional cell types of ovarian cancer cannot be clearly explained by the presence or absence of specific genetic changes [4]. Ovarian cancer is notoriously difficult to diagnose in its early stages. Consequently, approximately 90% of the patients with epithelial ovarian cancer are diagnosed with metastasis to the pelvis and upper abdomen and, for these patients, five year survival rates are less than 30% [1]. In contrast, the small proportion of patients diagnosed with stage I ovarian cancer (confined to the ovaries) have a five year survival rate in excess of 90% [1]. Even when patients with the same stage and histologic type of ovarian cancer are considered, the biologic factors that could predict disease behavior are not well understood. Thus, there is a pressing need to identify ways to distinguish and understand the different epithelial ovarian tumor subtypes in order to develop effective treatment strategies.

Human mitochondrial gene mutations have increasingly been associated with various cancers but with unclear pathophysiological significance [3,5-9]. Most mtDNA mutations occur in coding sequences because mtDNA lacks introns. The mitochondrial genome is more vulnerable to oxidative damage and undergoes a higher rate of mutation than does the nuclear genome [10,11]. Some of the mitochondrial genome aberrations found in cancer tissues are mutations in mtDNA-encoded Complexes I, III-V as well as mutations in the hypervariable regions [9]. MtDNA mutations in tumor cells are consistent with previous reports that tumor cells are subject to constitutive oxidative stress [9,11,12]. Although most of the reported mtDNA mutations in cancer are homoplasmic polymorphisms which are considered too subtle to cause any effect on oxidative phosphorylation (OXPHOS), long-term accumulation of subtle difference in OXPHOS activity may eventually result in oxidative stress [12,13]. Consequently, variations in the mtDNA may potentially play a role as a modifying risk factor in the development of age related diseases such as ovarian cancer. Mitochondrial aberrations have been identified in tumors of colorectal, breast, head and neck, kidney, lung, stomach and in the hematologic malignancies, leukemia and lymphoma [7,13-20]. Hence, we sought to determine the impact of mtDNA sequence variants in the pathogenicity of epithelial ovarian tumor subtypes.

Here we report the identification of mtDNA sequence variants in multiple specimens of ovarian carcinoma using a high-resolution restriction endonucleases and PCR-based sequencing analyses. We have found several highly frequent mtDNA sequence variants that could differentiate the three major histopathological subtypes and stages of serous, endometrioid and mucinous carcinomas of the ovary.

Methods

Subjects

One hundred and two frozen epithelial ovarian tumor tissues from three histologic subtypes (serous n = 42; endometrioid, n = 33; mucinous n = 17), each of the stages I-IV, benign cystadenomas, borderline tumors and 10 matched normal tissues that paired with the tumors were obtained from Southern Regional Cooperative Human Tissue Network and University of Alabama-Birmingham (UAB)-Ovarian Spore Center. The ovarian tumor subtypes and stages were histopathologically determined on the basis of the criteria outlined by the International Federation of Gynecology and Obstetrics (FIGO). All studies were implemented under Morehouse School of Medicine and UAB Institutional Review Board approval protocols. MtDNA was isolated from the frozen tissues using centrifugation method according to the manufacturer's protocols (BioVision, Research Products). Total mtDNA was quantified and diluted to 50 ng/μl for PCR reaction.

High Resolution Restriction Endonucleases and PCR-based Analysis of mtDNA variants

Extracted mtDNA from each tissue sample was PCR amplified using two large sets of overlapping primers. The first primer sets (*for-5'-CCGGGCCCATAACACTTGGG-3'*, position 16,453→16,472 in MITOMAP and *rev-5'-GGAGTGGGTTTGGGG CTAGG-3'* 1,696→1,677) yielded 1.8 kb fragment that flank the D-loop and part of 12 rRNA regions and the second primer sets (for-*5'-GGAT-GCCCCCCACCCTACC-3'*, 7,392→7,410 and *rev-5'-CCTT-GTGGTAAGAAGTGGGC-3'*, 8921-8902) yielded 1.5 kb fragments that spanned part of cytochrome oxidases I, II (COX I and II), ATPase 8 and ATPase 6 regions, which have been previously described [21,22]. The tRNAs enclosed in these regions are: Phe, Val, Ser, Asp, and Lys. The PCR was performed with 35 cycles of denaturation at 94°C for 1 minute, annealing at 59°C for 1 minute and extension at 72°C for 1 minute, and with the second set of primers, the annealing was 62°C for 1 minute. Amplified fragments were then purified using the QIAquick (QIAGEN) gel extraction kit. Each PCR product was digested with 14 restriction endonucleases (*AluI, AvaII, BamHI, DdeI, HaeII, HaeIII, HhaI, HincII, HinfI, HpaI, MspI, MboI, RsaI, and TaqI*) and were electrophoresis-gel analyzed side-by-side. Any mtDNA PCR fragments show-

ing differences in banding patterns between subtypes, stages of tumor and matched normal tissue samples were first directly sequenced to identify the exact nature of the new length variants detected by restriction analysis. Additionally, all the amplified products were sequenced with the same PCR primer sets, to assess the presence of other mtDNA mutations within the samples. PCR products were sequenced using ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Foster, CA).

Analysis of the mtDNA Sequences
The results of the mtDNA sequence analysis were compared with the published NCBI sequence (Accession # J01415). Furthermore, the sequence variants were compared with those in the mtDNA databank [3] to verify if the changes detected have previously been reported. Sequence alignment among subtypes and within stages were performed using Vector NTI advance 10-Align X program (Invitrogen). MtDNA sequence variants present in both the tumor and matched normal tissue were scored as germ-line variants. Any mtDNA sequence variant that were different between the tumor and the matched normal tissues were scored as somatic mutations. All sequence variants were confirmed by repeated analysis of the mtDNA extracted from the study samples.

Results and Discussion
The restriction analyses identified a number of band shifts indicating site gains or losses between and among ovarian subtypes and stages. However, these bands only indicated that the predicted sequence had changed. They did not identify specific base pair changes. We, therefore, used the original primers to sequence the band shifts in order to identify these specific changes. Among 102 ovarian tumor samples (including 10 paired tumor and matched normal tissues) that were sequenced, a total of three hundred and fifty-two (352) mtDNA variants were observed over a span of 3.3 kb fragment, including D-Loop, 12S rRNA-tRNA[phe], tRNA[val], COX I, tRNA[ser], tRNA[asp], COX II, tRNA-[lys], *ATPase 6* and *ATPase 8* (see additional file 1) and 238 of 352 (68%) were not previously reported [23]. Insertions and deletions accounted for 38.3% (135/352) of the observed mtDNA sequence variants suggesting instability of mtDNA in epithelial ovarian tumors and is consistent with previous studies by Gomez-Zaera *et al.*, [6] and Vega *et al.*, [19]. A high frequency of mtDNA mutations among our study samples, which have been previously reported [23] were apparent at np A263G (93/102), A1438G (95/102), A8860G (96/102) as shown in Table 1 and additional file 1. Also, there were high incidences (86–100%) 1648delT, T1653A/delT, 1659delT mtDNA sequence variants among the three epithelial ovarian tumor subtypes and stages compared with 100 human genome sequences [24] (Figure 1). MtDNA sequence variants T16519C, A73G and A263G were found at frequencies of 36%–95%

among our samples and these variants have been observed in various ethnic groups and other cancers [25]. A polymorphism, T16519C, has previously been shown to significantly increase breast cancer risk [26].

Consistent with other studies, we have observed the incidence of the polynucleotide repeat sequence at np 303–315, which has already been reported as a mutational hotspot D310, in a wide variety of human neoplasms [14,27-32]. The number of cytosine residues in the first stretch varied from C6-10T followed by the second stretch of C5-6 (Figure 2A). The reference sequence (mitomap-J01415) of this hot spot was C7TC5 and that of the general population with NCBI Blast Search was C7-8TC6 [33].

The c-stretch instability at np 303–315 was observed in 97% of the study samples, including the 10 tumor samples that paired with the matched normal tissues. This finding suggests that the observed c-stretch variants in ovarian tumors are germline origin. The instability at np 303 has been observed in some premalignant lesions of head and neck [16] and early stages of ovarian carcinomas [34], although none of the associations in those studies reached statistical significance. Perhaps the most striking observation was the incidence of TC insertion at np 310 found at a relatively high frequency of 81% (13/16) only in the early stages of serous subtype, which included benign cystadenomas 3/3, borderline tumors 4/4, stage I tumors 2/5, and matched normal tissues 4/4. While we could not conclusively rule-in poly C and the TC insertion instabilities as a major factor in the predominant subtype of epithelial ovarian tumorigenesis, certainly, the weight of the evidence, in spite of the limited sample size was not against it.

Different mechanisms have been put forward to explain the genomic instability of this stretch of cytosine residues [34-37]. The relative proportion of variable length polyC tracts appears to be actively maintained during cell division despite evidence of random mtDNA segregation, suggesting denovo regeneration of specific pattern following cell division by unknown molecular mechanisms [38,39]. It is unclear if this instability is associated with ethnicity, heredity or tissue specificity. Therefore, it is tempting to explore the significance of this D310 hot spot as a biomarker in detection, differentiation and progression of the various subtypes and stages of ovarian tumors.

Interestingly, we did observe three mtDNA mutations in the 12S rRNA gene at np A772T, 773delT, and 780delC in endometrioid stage III tumors, of which 773delT (7/7) and 780delC (7/7) with a frequency of 100% in endometrioid stage IIIC has not been reported. Furthermore, two mutations of interest were observed only in serous sub-
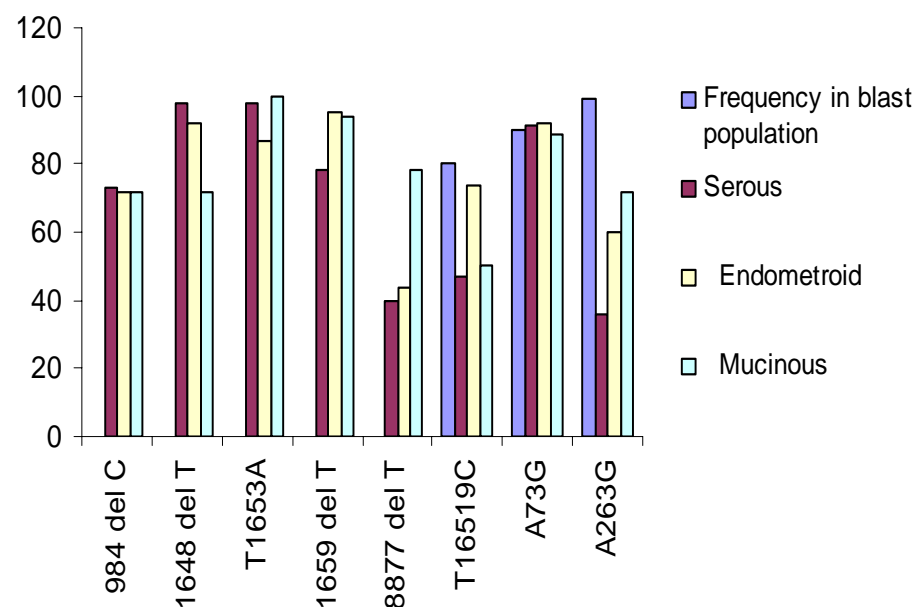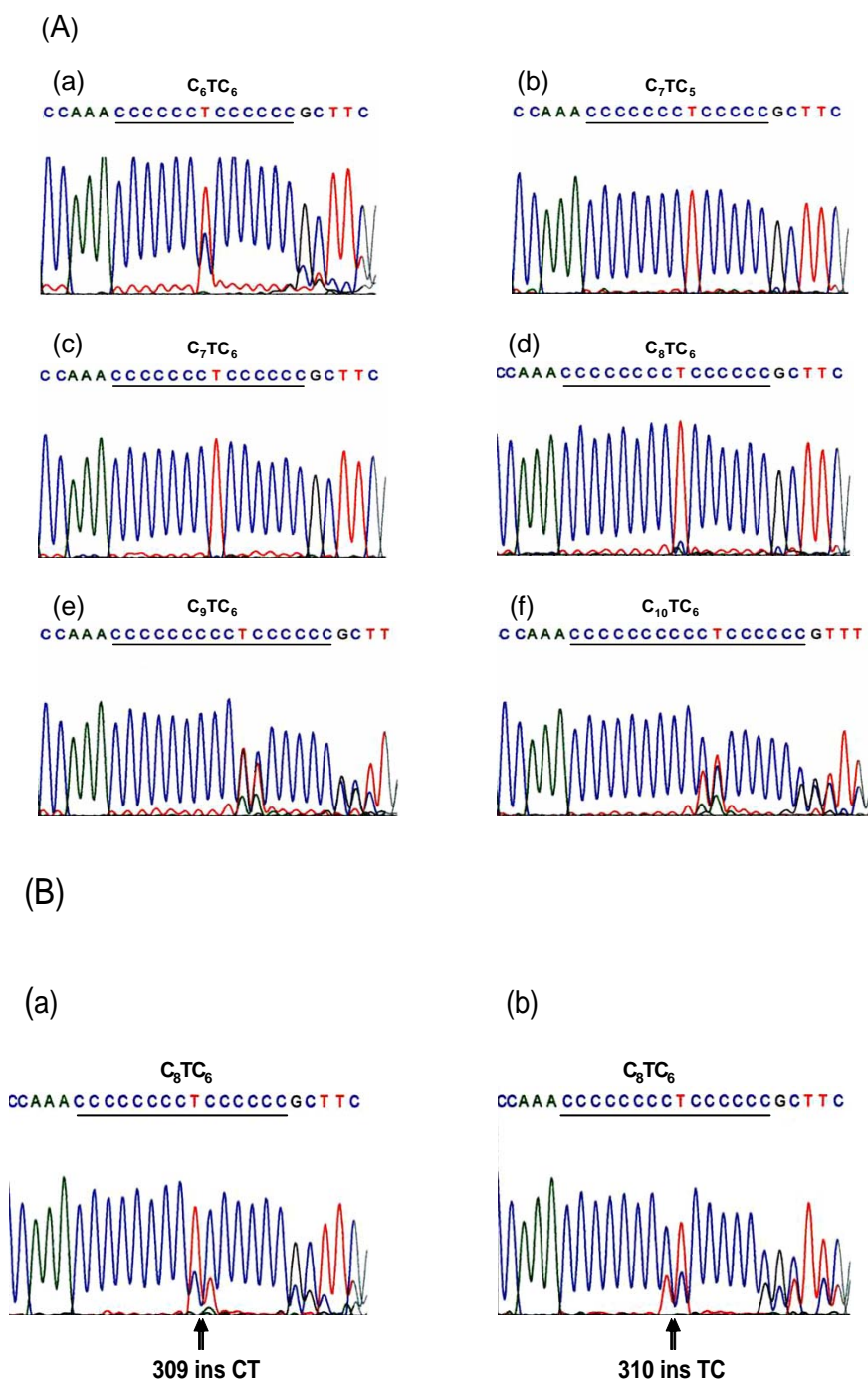
Figure 1
Distributions of the most frequent mtDNA mutations (984delC-*12S rRNA*; 1648delC, T1653A, 1659delT-*tRNA^val*; 8877delC-*ATPase 6*; T16519C, A73G, A263G-D-loop region) in three epithelial ovarian tumor subtypes compared to 100 human genome sequences [24]. Mutation A263G appears to be less frequent in the epithelial ovarian tumor subtypes compared to the general population and the mtDNA-*tRNA^val* 1653delT variant may be considered as a causative event for the three epithelial ovarian tumor subtypes.

Table 1: High frequency mtDNA variants among three epithelial ovarian tumor subtypes

Mitochondrial genes/regions	Nucleotide Position np	Mutation Frequency Total # of cases 102	% frequency in subtypes		
			Serous	Endometrioid	Mucinous
D-loop	16487 **del A**[1]	73	87	69	39
D-loop	T16519C	52	47	74	50
D-loop	A73G	59	91	92	89
D-loop	A263G	93	36	60	72
12S rRNA	**984 del C**[1]	**58**	**73**	**72**	**72**
	C984G[1]	**16**			
12S rRNA	A1438G	95	91	95	94
tRNA^val	1648 **del T**[1]	93	98	92	72
tRNA^val	**1653 del T**[1]	**20**	**98**	**87**	**100**
	T1653A[1]	**76**			
tRNA^val	1659 **del T**[1]	89	78	95	94
COX2	8237 **del A**[1]	68	82	49	67
ATP6	A8860G	96	96	97	83
ATP6	8877 **del T**[1]	49	40	44	78
ATP6	**T8889 A**[1]	**51**	**93**	**87**	**83**
	T8889C[1]	**16**			
	8889del T[1]	**24**			

[1] Not reported in mtDNA databank.
The frequency % of the three subtypes at np 984, 1653 and 8889 represents the sum total of **the different types** of mutations occurring at that position.

Figure 2
A. MtDNA sequence electropherograms showing variations in consecutive C-stretch at np 303–310 of the D-loop region obtained from the three subtypes of the epithelial ovarian neoplasms (a) C_6TC_6 and (b) C_7TC_5 sequences from stage I of endometrioid tumor; (c) C_7TC_6 and (d) C_8TC_6 sequences from stage I of mucinous tumor; (e) C_9TC_6 sequence obtained from stage III of mucinous tumor; (f) $C_{10}TC_6$ sequence from stage III of serous tumor. Interestingly, the C-stretch instability at np 303–315 was observed in 97% of our study samples. B. Sequence electropherograms showing the mtDNA instability at np (a) 309insCT in stage II of endometrioid tumor and (b) 310insTC observed only in early stages of serous subtype in this study. Arrow shows the unique 309insCT and 310insTC patterns respectively.

type, at np 1657delC in stage IV (10/10) and 8221delA in benign cystadenomas (3/3) and borderline tumors (4/4). To our knowledge, the incidence of specific mtDNA variants in association with FIGO stages and subtypes of epithelial ovarian tumor have not been reported.

A previous study by Liu *et al.*, [40] reported a high incidence of somatic mtDNA mutations in ovarian carcinoma and it is consistent with our observation where we observed somatic mutations in the mtDNA of a majority of the tumor samples. In contrast to the same study [40], we did not find an increase or decrease of CA repeats at 514–524, except few ins/del sequence variants with no significant association with specific ovarian tumor stages, subtypes or matched normal tissues. Perhaps the discrepancies may be attributed to the histopathologic stages of the tumor tissues that we analysed and the mtDNA haplogroup within ovarian cancer.

Conclusion
Notably, most of the identified mtDNA sequence variants occurred in D-loop region and any mutation in this region may potentially modify the rate of mtDNA replication. The D-loop region is important for replication and expression of mitochondrial genome due to the leading-strand origin of replication and the promoters of transcription [41]. Although our study lacks functional data and clinical outcome of the mitochondrial genome alterations in relationship to ovarian neoplasms, it suggests that mtDNA sequence variants may play a role in etiological differences that may exist between the pathogenicity of subtypes and stages of benign and invasive epithelial ovarian tumors. Large population-based studies are required to precisely quantify the functional role of mtDNA sequence variants in histological subtypes and stages of ovarian cancer. Given that we only sequenced 3.3 kb of 16.5 kb fragment of mitochondrial genome, the level of mtDNA sequence variants that could be correlated with ovarian tumor subtypes and stages may far exceed the number we have observed in this study.

Competing interests
The author(s) declare that they have no competing interests.

Authors' contributions
FOA conceived, designed, coordinated the study, and participated in data analysis and drafted the manuscript. MS participated in acquisition and analysis of the data and helped to draft the manuscript. KK participated in acquisition and analysis of the data and helped to draft the manuscript. MZ participated in acquisition of the data. MO participated in the review of the manuscript. KO helped to draft the manuscript. EP provided some of the ovarian tumor samples and participated in the review of the manuscript. All authors read and approved the final manuscripts.

Additional material

Additional file 1
Mitochondrial DNA mutations in epithelial ovarian tumors. The data provided represent the mitochondrial sequence variants spanning 3.3 kb fragment that comprised the D-Loop and 12S rRNA-tRNA^{phe}, tRNA^{val}, tRNA^{ser}, tRNA^{asp}, tRNA^{lys}, ATPase 6, ATPase 8, cytochrome oxidase I and II genes. in our study epithelial ovarian tumor samples.
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References
1. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ: **Cancer statistics.** *CA Cancer J Clin* 2005, **55**:10-30.
2. Bell DA: **Ovarian surface epithelial-stromal tumors.** *Hum Pathol* 1991, **22**:750-762.
3. Scully RE: **Pathology of ovarian cancer precursors.** *J cell Biochem Suppl* 1995, **23**:208-218.
4. Ono K, Tanaka T, Tsunoda T, Kitahara O, Kihara C, Okamota A, Ochiai K, Takagi T, Nakamura Y: **Identification by cDNA microarray of gene involved in ovarian carcinogenesis. 2000.** *Cancer Res* 2001, **60**:5007-11.
5. Nishikawa M, Nishiguchi S, Shiomi S, Tamori A, Kho N, Takeda T, Kubo S, Hirohashi K, Kinoshita H, Sato E, Inoue M: **Somatic mutation of mitochondrial DNA in cancerous and noncancerous liver tissue in individuals with hepatocellular carcinoma.** *Cancer research* 2001, **61**:1843-1845.
6. Gomez-Zaera M, Abril J, Gonzalez L, Aguilo F, Condom E, Nadal M, Nunes V: **Identification of somatic and germline mitochondrial DNA sequence variants in prostate cancer patients.** *Mutation Research* 2006, **595**:42-51.
7. Aikhionbare FO, Khan M, Carey D, Okoli J, Go R: **Is cumulative frequency of mitochondrial DNA variants a biomarker for colorectal tumor progression?** *Molecular Cancer* 2004. DOI:10.1186/1476-4598-3-30
8. Petros JA, Baumann AK, Ruiz-Pesini E, Amin MB, Sun CQ, Hall J, Lim S, Issa MM, Flanders WD, Hosseini SH, Marshall FF, Wallace DC: **mtDNA mutations increase tumorigenicity in prostate cancer.** *PNAS* 2005, **102(3)**:719-24.
9. Penta JS, Johnson FM, Wachsman JT, Copeland WC: **Mitochondrial DNA in human malignancy.** *Mutat Res* 2001, **488**:119-133.
10. Yakes FM, Van Houten B: **Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress.** *Proc Natl Acad Sci USA* 1997, **94**:514-9.
11. Marcelino LA, Thilly WG: **Mitochondrial mutagenesis in human cells and tissues.** *Mutat Res* 1999, **434**:177-203.
12. Toyokuni S, Okamoto K, Yodoi J, Hiai H: **Persistent oxidative stress in cancer.** *FEBS lett* 1995, **358**:1-3.
13. Polyak K, Li Y, Zhu H, Lengauer C, Wilson JK, Markowitz SD, Trush MA, Kinzler KW, Vogelstein B: **Somatics mutations of the mitochondrial genome in human colorectal tumours.** *Nat Genet* 1998, **20**:291-293.
14. Richard SM, Bailliet G, Paez GL, Bianchi MS, Peltomaki P, Bianchi NO: **Nuclear and mitochondrial genome instability in human breast cancer.** *Cancer Res* 2000, **60**:4231-7.

15. Tamura G, Nishizuka S, Maesawa C, Suzuki Y, Iwaya T, Sakata K, Endoh Y, Motoyama T: **Mutations in mitochondrial control region DNA in gastric tumuors of Japanese patients.** *Eur J cancer* 1999, **35**:316-319.
16. Ha PK, Tong BC, Westra WH, Sanchez-Cespedes M, Parrella P, Zahurak M, Sidransky D, Califano JA: **Mitochondrial C-tract alternation in premalignant lesions of the head and neck. a marker for progression and clonal proliferation.** *Clin Cancer Res* 2002, **8**:2260-2265.
17. Nagy A, Wilhelm M, Kovacs G: **Mutations of mtDNA in renal cell tumours arising in end-stage renal disease.** *J Pathol* 2003, **199**:237-242.
18. Matsuyama W, Nakagawa M, Wakimoto J, Hirotsu Y, Kawabata M, Osame M: **Mitochondrial DNA mutation correlates with stage progression and prognosis in non-small cell lung cancer.** *Hum Mutat* 2003, **21**:441-443.
19. Vega A, Salas A, Gamborino E, Sobrido MJ, Macaulay V, Carracedo A: **mtDNA mutations in tumors of the central nervous system reflect the neutral evolution of mtDNA in populations.** *Oncogene* 2004, **23**:1314-1320.2.
20. Fliss MS, Usadel H, Caballero OL, Wu L, Buta MR, Eleff SM, Jen J, Sidransky D: **Facile detection of mitochondrial DNA mutations in tumors and bodily fluids.** *Science* 2000, **287**:2017-2019.
21. Torroni A, Shurr TG, Yang CC, Szathmary EJ, Williams RC, Schanfield MS, Troup GA, Knowler WC, Lawrence DN, Weiss KM: **Native American mitochondrial DNA analysis indicates that the Amerind and Nadene populations were founded by two independent migrations.** *Genetics* 1992, **130**:153-162.
22. Watson B Jr, Khan MA, Desmond RA, Bergman S: **Mitochondrial DNA mutations in black Americans with hypertension associated end-stage renal disease.** *Am J Kidney Dis* 2001, **38(3)**:529-536.
23. **MITOMAP A human mitochondrial genome database** [http://www.mitomap.org]
24. **National Center for Biotechnology Information BLAST** 2001 [http://www.ncbi.nlm.nih.gov/blast/].
25. Krurtz A, Lueth M, Kluwe L, Zhang T, Foster R, Mautner V-F, Hartmann M, Tan D-T, Martuza RL, Friedrich RE, Driever PH, Wong L-JC: **Somatic mitochondrial DNA mutations in neurofibromatosis type 1-associated tumors.** *Mol Cancer Res* 2004, **2(8)**:433-441.
26. Tan D-J, Bai R, Wong L-JC: **Comprehensive scanning of somatic mitochondrial DNA mutations in breast cancer.** *Cancer Res* 2002, **62**:976-976.
27. Sanches-Cespedes M, Parrella P, Nomoto S, Cohen D, Xiao Y, Etseller M, Jeronimo C, Jordan RC, Nicol T, Koch WM, Schoenberg M, Mazzarelli P, Fazio VM, Sidransky D: **Identification of a mononucleotide repeat as a major target for mitochondrial DNA alteration in human tumors.** *Cancer Res* 2001, **61**:7015-7019.
28. Aral C, Kaya H, Çelikel CA, Akkiprik M, Sönmez O, Güllüoğlu BM, Özer A: **A novel approach for rapid screening of mitochondrial D310 polymorphism.** *BMC Cancer* 2006, **6**:21.
29. Parrella P, Xiao Y, Fliss M, Sanchez-Cespedes M, Mazzarelli P, Rinaldi M, Nicol T, Gabrielson E, Cuomo C, Cohen D, Pandit S, Spencer M, Rabitti C, Fazio VM, Sidransky D: **Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates.** *Cancer Res* 2001, **61**:7623-7626.
30. Parrella P, Seripa D, Matera MG, Rabitti C, Rinaldi M, Mazzarelli P, Gravina C, Gallucci M, Altomare V, Flammia G, Casalino B, Benedetti-Panici PL, Fazio VM: **Mutations of D310 mitochondrial mononucleotide repeat in primary tumors and cytological specimens.** *Cancer Lett* 2003, **190**:73-77.
31. Tong BC, Ha PK, Dhir K, Xing M, Westra WH, Sidransky D, Califano JA: **Mitochondrial DNA alterations in thyroid cancer.** *J Surg Oncol* 2003, **82**:170-173.
32. Isaacs C, Cavalli LR, Cohen Y, Pennanen M, Shankar LK, Freedman M, Singh B, Liu M, Gallagher A, Rone JD, Dickson RB, Sidransky D, Haddad BR: **Detection of LOH and mitochondrial DNA alterations in ductal lavage and nipple aspirate fluids from high-risk patients.** *Breast Cancer Res Treat* 2004, **84**:99-105.
33. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *J Mol Biol* 1990, **215**:403-410.
34. Wang Y, Liu VW, Tsang PC, Chiu PM, Cheung AN, Khoo US, Nagley P, Ngan HY: **Microsatellite instability in mitochondrial genome of common female Cancers.** *Int J Gynecol Cancer* 2006, **16(Suppl 1)**:259-266.
35. Mambo E, Gao X, Cohen Y, Guo Z, Talalay P, Sidransky D: **Electrophile and oxidant damage of mitochondrial DNA leading to rapid evolution of homoplasmic mutations.** *Proc Natl Acad Sci USA* 2003, **100**:1838-43.
36. Longley MJ, Nguyen D, Kunkel TA, Copeland WC: **The fidelity of human DNA polymerase gamma with and without exonucleolytic proofreading and the p55 accessory subunit.** *J Biol Chem* 2001, **276**:38555-62.
37. Graziewicz MA, Day BJ, Copeland WC: **The mitochondrial DNA polymerase as a target of oxidative damage.** *Nucleic Acids Res* 2002, **30**:2817-24.
38. Malik S, Sudoyo H, Pramoonjago P, Sukarna T, Darwis D, Marzuki S: **Evidence for the de novo regeneration of the pattern of the length heteroplasmy associated with the T16189C variant in the control (D-loop) region of mitochondrial DNA.** *J Hum Genet* 2002, **47**:122-130.
39. Shin MG, Kajigaya S, Tarnowka M, McCoy JP Jr, Levin BC, Young NS: **Mitochondrial DNA sequence heterogeneity in circulating normal human CD34 cells and granulocytes.** *Blood* 2004, **103**:4466-4477.
40. Liu VW, Shi HH, Cheung AN, Chiu PM, Leung TW, Nagley P, Wong LC, Ngan HY: **High incidence of somatic mitochondrial DNA mutations in human ovarian carcinomas.** *Cancer Res* 2001, **61**:5998-6001.
41. Taanman JW: **The mitochondrial genome: structure, transcription, translation and replication.** *Biochim Biophys Acta* 1999, **1410**:103-123.